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Twenty-ninth day of August 2003

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AUSTRALIA

Patents Act 1990

Proteome Systems Ltd

PROVISIONAL SPECIFICATION

Invention Title:

Method for diagnosing disorders

The invention is described in the following statement:

METHOD FOR DIAGNOSING DISORDERS

The present invention relates to the diagnosis of abnormal physiological conditions, such as diseases, using glycosylation profiles generated from biological samples. The present invention also relates to the identification of glycan diagnostic markers of abnormal physiological conditions and therapeutic targets.

Summary of the invention

The present invention aims to provide improved means and methods for the diagnosis of disease, based upon changes in the glycosylation profile in certain disease states. This is achieved by treatment of glycoconjugate molecules from a biological sample of interest to release glycans and analysis of the released glycans by mass spectrometry to produce a profile which can then be compared with a control profile. Advantageously, partial enrichment/resolution of glycoconjugate molecules is performed prior to release of glycans.

The advantage of this method is that it is not necessary to know the identity of the glyconjugates from which the glycans are derived nor is it necessary to purify individual molecules since a collective glycan profile can being obtained from a plurality of molecules to provide a representation of glycosylation changes. Furthermore, an important feature of this method is that the glycans are separated from the other constituent parts of the molecule prior to analysis by mass spectrometry and therefore the profile is derived from the released glycans and not the complete glycoconjugates. This provides a less complex profile than if intact glycosylated molecules are subjected to mass spectrometry.

We have demonstrated the applicability of this technique to the detection of ovarian cancer and the monitoring of cystic fibrosis using plasma samples and sputum samples, respectively.

Thus, in a first aspect, the present invention provides a method for identifying the presence of an abnormal physiological condition in an individual, which method comprises:

(i) providing a biological sample from the individual;

(ii) treating one or more glycoconjugates present in the sample to release glycans;

(iii) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(iv) analysing the glycosylation profile for changes in a glycan marker which is indicative of the abnormal physiological condition.

In preferred embodiment, the biological sample is subjected to one or more separation steps to resolve one or more glycoconjugates from other components in the sample prior the treatment of the glycoconjugates to release glycans.

Accordingly, the present invention also provides a method for identifying the presence of an abnormal physiological condition in an individual, which method comprises:

(i) providing a biological sample from the individual;

(ii) subjecting the sample to one or more separation steps to resolve one or more glycoconjugates from other components in the sample;

(iii) treating the one or more glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) analysing the glycosylation profile for changes in a glycan marker which is indicative of the abnormal physiological condition.

Preferably step (v) comprises comparing the glycosylation profile produced in step (iv) with a control glycosylation profile.

Preferably, the one or more separation steps are selected from electrophoresis, such as gel electrophoresis, and chromatography.

Typically, step (iii) comprises releasing the glycans from the glycoconjugates by enzymatic and/or chemical means.

The glycoconjugate is preferably selected from a glycoprotein, a proteoglycan and a glycolipid.

Preferably, the abnormal physiological condition is a disease state, such as a pathogenic infection, the presence of a malignancy or a respiratory disorder e.g. cystic fibrosis.

In a preferred embodiment, a plurality of glycoconjugates are treated to release glycans.

In one embodiment, in step (ii) substantially the total glycoconjugate content of the sample is separated from other components in the sample and treated to release glycans. Preferably, substantially the total glycoprotein and/or proteoglycan content is separated from other components in the sample and treated to release glycans.

In an alternative embodiment, not all of the glycoconjugates present in the sample are treated to release glycans.

The method of the first aspect of the invention may further comprise identifying and characterising one or more glycans having altered levels in the biological sample from the individual as compared with a control sample.

The method of the first aspect of the invention may be carried out repeatedly over a period of time so as to monitor the development of a particular disorder, e.g. to monitor the progress of a disease, such as an infection, and to assist in determining the optimum clinical regimen for a given patient.

In addition to the diagnosis of disorders, the glycan profiling method of the present invention can be used to monitor the response to treatment of the disorder.

Accordingly, in a second aspect, the present invention provides a method for monitoring the efficacy of a therapeutic treatment of an abnormal physiological condition in an individual, which method comprises:

(i) providing a biological sample from the individual undergoing the therapeutic treatment;

(ii) subjecting the sample to one or more separation steps to resolve one or more glycoconjugates from other components in the sample;

(iii) treating the one or more glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) analysing the glycosylation profile for changes in a glycan marker which is indicative of the abnormal physiological condition.

Preferably, step (v) comprises comparing the glycosylation profile with a profile obtained with a biological sample from the individual prior to commencement of the treatment and/or at an earlier time point in the treatment.

In addition to diagnosis/monitoring of treatment, the glycan profiling method of the present invention may be used to identify specific glycans or groups of glycans whose levels are altered in particular disorders.

Accordingly, in a third aspect, the present invention provides a method for identifying a glycan whose levels are altered in an individual suffering from an abnormal physiological condition, which method comprises:

(i) providing a biological sample from the individual;

(ii) subjecting the sample to one or more separation steps to resolve one or more glycoconjugates from other components in the sample;

(iii) treating the one or more glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) comparing the profile with a control profile.

(vi) identifying a glycan whose levels are altered in the profile obtained in step (iv) as compared with the control profile.

In a related aspect, the present invention provides a method for identifying a glycoconjugate whose levels are altered in an individual suffering from an abnormal physiological condition, which method comprises:

(i) providing a biological sample from the individual;

(ii) subjecting the sample to one or more separation steps to resolve one or more glycoconjugates from other components in the sample;

(iii) treating the one or more glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) comparing the profile with a control profile.

(vi) identifying a glycan whose levels are altered in the profile obtained in step (iv) as compared with the control profile; and

(vii) identifying a glycoconjugate present in the biological sample from which the glycan is derived.

In a fourth aspect, the present invention provides a method for identifying a glycan which is a diagnostic marker for an abnormal physiological condition, which method comprises:

(i) providing a biological sample from an individual suffering from an abnormal physiological condition;

(ii) subjecting the sample to one or more separation steps to resolve one or more glycoconjugates from other components in the sample;

(iii) treating the one or more glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) identifying a glycan whose levels are altered in the profile obtained step (iv) as compared with a control profile, the identified glycan being the diagnostic marker.

In a related aspect, the present invention provides a method for identifying a glycoconjugate which is a diagnostic marker for an abnormal physiological condition, which method comprises:

(i) providing a biological sample from an individual suffering from an abnormal physiological condition;

(ii) subjecting the sample to one or more separation steps to resolve one or more of glycoconjugates from other components in the sample;

(iii) treating the one or more of glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile;

(v) identifying a glycan whose levels are altered in the profile obtained step (iv) as compared with a control profile; and

(vi) identifying a glycoconjugate present in the biological sample from which the glycan is derived, the identified glycoconjugate being the diagnostic marker.

The glycan profiling method of the present invention may also be used to identify glycans that can be targeted on a therapeutic basis. For example, a glycan derived from a pathogen glycoconjugate may be used to generate inhibitory molecules that compete with the glycoconjugate for binding to a host molecules.

Accordingly, in a fourth aspect, the present invention provides a method for identifying a candidate therapeutic target, which method comprises:

(i) providing a biological sample from an individual suffering from an abnormal physiological condition;

(ii) subjecting the sample to one or more separation steps to resolve one or more of glycoconjugates from other components in the sample;

(iii) treating the one or more of glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) identifying a glycan whose levels are altered in the profile obtained in step (iv) as compared with a control profile, the identified glycan being the identified candidate therapeutic target.

In a related aspect, the present invention provides a method for identifying a candidate therapeutic target, which method comprises:

(i) providing a biological sample from an individual suffering from an abnormal physiological condition;

(ii) subjecting the sample to one or more separation steps to resolve one or more glycoconjugates from other components in the sample;

(iii) treating the one or more of glycoconjugates to release glycans;

(iv) analysing the released glycans by mass spectrometry to produce a glycosylation profile; and

(v) identifying a glycan whose levels are altered in the profile obtained in step (iv) as compared with a control profile; and

(vi) identifying a glycoconjugate present in the biological sample from which the glycan is derived, the identified glycoconjugate being the identified candidate therapeutic target.

In the second to fourth aspects of the invention, step (ii) is a preferred step but may be omitted as in the first aspect.

The present invention also provides an oligosaccharide of the formula Hex-HexNAcol+NeuAc+$NeuAc_2$. (structure 14 of Table 1). The present invention further provides a method of detecting ovarian cancer in a human patient which method comprises performing the method of the first aspect of the invention and determining the presence of one or more oligosaccharides set out in Table 1, preferably one or more oligosaccharides having the structures selected from structures 1, 2, 3, 6, 7, 8, 14, 19 and 20 set out in Table 1.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in biochemistry). Standard techniques are used for biochemical methods and chemical methods.

Production of glycan profiles and diagnosis/monitoring of treatment efficacy

1. Sample resolution

In a preferred embodiment of the method of the first aspect, a biological sample from a subject is subjected to one or more separation steps to resolve one or more of glycoconjugates present in the sample from other components in the sample. However, this step is not essential, and biological samples may be treated to cleave glycans from glycoconjugates without any separation steps.

The term "glycoconjugates" refers to any molecule comprising covalently linked sugar moieties linked to non-sugar moieties. Particular examples include glycoproteins, proteoglycans and glycolipids. Carbohydrates per se, i.e. not linked to non-sugar moieties, are preferably excluded.

Any biological sample comprising glycoconjugates may be used in the method of the present invention. Examples of suitable samples include blood, serum, sputum, tears, urine, saliva, breast milk, and nasal secretions, or fractions thereof. The nature of the biological sample chosen will vary depending on the type of condition that is the subject of the diagnostic/prognostic test.

The subject may be any animal, preferably a mammal, and most preferably a human. The subject may also be a non-human animal.

The sample may be treated to remove debris and/or insoluble biological material. For example, samples may be centrifuged and the supernatant used for subsequent testing. Typically, this is carried out as a pre-treatment step prior to subjecting the sample to one or more separation steps to resolve the glycoconjugates,

Separation steps include any technique that involves the separation and/fractionation of the components of the biological sample. Particularly preferred separation methods are electrophoresis or chromatography.

Electrophoresis methods include gel electrophoresis, for example gel electrophoresis using polyacrylamide gels. Electrophoretic separation may be effected on the basis of molecular weight (size) and/or charge. A preferred method of separation on the basis of charge is isoelectric focussing (IEF), preferably using immobilized pH gradients (IPGs). The pH gradients may be narrow (e.g. 1 pH unit or less) or wide (e.g. from pH 2 to pH 12).

It is particularly preferred to use a 2D method where IPG-IEF is used for the first dimension and SDS-PAGE is used for the second dimension.

Glycoconjugates may be recovered from the gels using standard techniques, for example the proteins may be transferred to PVDF membranes by electroblotting and portions of the membrane cut out and used for the subsequent analysis steps. Optionally, gels/blots may be stained with reagents that identify sugars, such as Periodic Acid/Schiffs base (PAS).

Depending on the nature of the sample and the electrophoretic technique used, bands/spots identified on the gel will contain more than one glycoconjugate. Where the resolution is such that bands/spots are likely to contain only one molecular species, a number of bands/spots may be picked if it desired to profile a plurality of glycoconjugates.

Chromatographic techniques include separations based on size or charge e.g. gel filtration, size exclusion chromatography, ion-exchange chromatography, and separations based on binding to specific ligands i.e. affinity chromatography. Since the method of the invention involves the resolution of glycoconjugates, an affinity chromatography matrix material that comprises lectins may be particularly convenient since many glycoconjugates will bind to the lectins, leaving other cellular components to be washed through. The purified glycoconjugates may then be recovered with a suitable eluting agent.

Chromatography may be conducted in batch or using columns. Chromatographic fractions may or may not be combined. Optionally, fractions may be concentrated prior to subsequent glycan removal and analysis.

Where the separation technique is designed to resolved glycolipids, a preferred separation technique is thin layer chromatography.

The separation methods need not resolve the glycoconjugates into individual molecular species since the diagnostic method of the invention may advantageously be used to profiling a plurality of glycoconjugates, for example at least 5, 10 or 15 separate species.

2. Glycan cleavage

The glycans are released from the one or more resolved glycoconjugates using any suitable technique, such as enzymatic and/or chemical means. Specific enzymes that may be used include enzymes that cleave *N*-linked oligosaccharides (e.g. PNGaseF). Chemical means include the release of O-linked oligosaccharides by reductive beta-elimination.

Released glycans are preferably subjected to a purification step to remove reagents, salts, proteins etc., for example using a graphitised carbon column as described in our earlier US Patent No. 6376663.

3. Mass spectrometry, profile generation and analysis

The glycans are then analysed by mass spectrometry to produce a glycosylation profile. Suitable mass spectrometry techniques include MALDI-TOF, liquid-chromatography mass spectrometry, electrospray ionisation (ESI), nano electrospray ionisation (nESI) and tandem mass spectrometry.

The resulting profile provides information such as the molecular weight, abundance and/or structure of one or more released oligosaccharides. Any one or more of these characteristics, which differs between the normal and abnormal

states, may be used a marker which is indicative of an abnormal conditions. Typically a plurality of differences are used as the basis of a diagnosis, for improved accuracy.

Once the glycosylation profile has been produced, the profile is analysed to identify the presence of a marker which differs from the normal state. Typically, this step of the method involves comparing the glycosylation profile produced from the biological sample of the subject with a control glycosylation profile. The control profile may be generated at the same time as the sample profile or may be a control profile generated previously. Where a profile has been generated previously and a plurality of marker glycan species identified, it may only be necessary to analyse the sample profile for the presence or absence of those particular marker species.

Preferably, the control glycosylation profile is produced from released oligosaccharides obtained from a biological sample of a subject known to be free of the disorder.

A glycan marker is a characteristic, in terms of molecular weight, abundance or structure, which is different between the glycosylation profile of (i) and (ii). It is apparent that a plurality of glycan markers may be identified for any particular disease.

A number of samples may be taken over a period of the time and profiles generated so as to monitor the development of a particular disorder, to monitor the progress of a disease, such as an infection. The changes in particular glycan markers can be used to monitor the progress of the development of the disorder and assist clinicians in determining the best clinical practice with respect to an individual patient. This monitoring process may be carried out prior to initiation of treatment and/or as described below, during treatment.

In another embodiment, the glycan profile is used to monitor the progress/efficacy of a treatment regimen. For example, our results show that the glycan profile obtained from a cystic fibrosis patient, with a bacterial infection, following antibiotic treatment differed from the profile obtained prior to treatment. Indeed, the glycan profile obtained from the cystic fibrosis patient following antibiotic treatment became more similar to the profile obtained from a normal patient.

Consequently, the profiling method of the present invention can be used in a similar manner to that described above for diagnosis, to monitor therapeutic treatments by repeated sampling and glycan profiling. Samples are typically taken at one or more time points during treatment and the resulting profile

compared with a profile obtained from a sample taken before treatment commenced or at an earlier time point. The changes in particular glycan markers can be used to monitor the progress of the treatment and/or to provide clinicians with a prognosis of the likely outcome of a particular treatment regimen.

The method of the present invention is applicable to diseases or other abnormal physiological conditions which are characterised by altered glycosylation patterns compared to the normal state. Such diseases include cancer, pathogenic infection, autoimmune disease or inflammatory disease.

The cancer may be a carcinoma, sarcoma or lymphoma, including cancer of the ovaries, breast, lung, prostrate, liver, brain, urinary tract, pancreas, blood cells, bone marrow, and lymph nodes.

The inflammatory disease is preferably associated with pathogenic infection such as pulmonary bacterial infection, which may be caused by one or more of *Pseudomonas aeruginosa, Hemophilus influenza* and *Staphylococcus aureus*. Preferably, the pathogenic infection and/or inflammatory disease is one which is associated with a respiratory disorder, for example a disorder characterised by excessive mucus production. Excess mucus production may be due to some physiological defect in the patient, such as in the case of cystic fibrosis, or may be caused by infection by pathogens such as viruses or bacteria, as in the case of tuberculosis or bronchial infections. The excess mucus production may render the patient more susceptible to subsequent bacterial infection. Furthermore, primary and/or secondary infections may result in inflammation mediated by the patient's immune system. Most preferably, the disease is cystic fibrosis, tuberculosis, Crohn's disease, ulcerative colitis or bronchial infections including chronical bronchitis.

As discussed above, the glycoconjugates from which the profiled glycans are derived include glycoproteins (for example mucins), proteoglycans, and glycolipids (including gangliosides). One or more of the glycans may comprise N-acetyl glucosamine, N-acetyl galactosamine, mannose, glucose, galactose, xylose, sialic acid, sulphate, phosphate and/or fucose residues. Preferably, one or more of the glycans comprises sialic acid, sulphate, phosphate and/or fucose residues.

In one embodiment, the glycoconjugates are endogenous to the subject, meaning that they are synthesised by the subject's cells and are not merely present due to infection or foreign bodies. Typically, the glycoconjugates are naturally present on the surface of host cells of the subject and/or are secreted by host cells into bodily fluid. In respiratory disorders such as cystic fibrosis or

bronchial infections, the endogenous glycans to be analysed will typically be those present on cells in the mucosal lining of the lungs, or secreted into the mucus lining the lungs.

Of particular interest in the present invention are the glycan portions of the glycoconjugates which are targeted by pathogens and/or host immune cells, and/or which are bound by proteins, such as receptors, present on the surface of said pathogens and immune cells. Herein, this portion is referred to as the epitope of the glycan. In the case of mucin, the epitopes may include Le^a, Le^y, Le^x, sialyl-Le^x and 3'-sulpho-Le^x.

Identification of glycans and glycoconjugates associated with disorders

The methods of the invention may be used to identify glycans and their associated glycoconjugates whose levels or mass are altered in subjects with a particular disorder.

The techniques for identifying glycan markers differ from the first aspect of the invention only in that prior to the analysis, the markers have not yet been identified and therefore it is essential to include appropriate control samples so that a comparison can be conducted for any molecular species that vary between the sample being tested and the controls.

Typically, a number of sample profiles are compared with a number of control profiles to identify molecular species whose abundance varies between the control profiles and the sample profiles.

Once one or more markers have been identified, it may be desirable to characterise the structure of the glycan using standard techniques, such as MS/MS. Furthermore, the glycoconjugate from which the glycan is derived may also be identified. This is conveniently achieved in the case of profiles produced from glycoconjugates resolved by 2D gel electrophoresis by reference to the spots on the gel that were used to generate the profile. For example, a spot on the gel that gave rise to a particular glycan marker may be analysed by mass spectrometry, following protease digestion, with or without a glycan cleavage step, to identify the protein part of the glycoconjugate.

The results described herein illustrate the identification and characterisation of specific glycan markers associated with ovarian cancer. These specific markers include the two isomers NeuAcα2-6(Galβ-3)GalNAcol (galactosylated Sialyl-Tn antigen)(structure 1) and NeuAcα2-3Galβ1-3GalNAcol (Sialyl T-antigen) (structure 2) found together with a disialylated structure

NeuAcα2-6(NeuAcα2-3)Galβ-3)GalNAcol (Disialyl T-antigen)(structure 6) – see table 1.

A particularly interesting trisialylated oligosaccharide structure was found. It was detected with an $[M-H]^-$-ion at *m/z* 1257.2 ($Hex_1HexNAc_1NeuAc_3$) (structure 14 – see table 1) at a level of 1 % in all the samples analysed.

Thus the presence of these markers in profiles obtained by the methods of the present invention may be used to diagnose ovarian cancer in a patient.

The identification of glycan markers and optionally the glycoconjugates from which they are derived also provides a means for identifying potential therapeutic targets. Thus, once a glycan marker has been identified as described in the third aspect of the invention, it may be used to identify potential therapeutic agents for the prevention or treatment of the disorder with which it is associated.

The various features and embodiments of each aspect of the present invention apply, as appropriate, to the other aspects, *mutatis mutandis*. Consequently features specified in one aspect may be combined with features specified in other aspects, as appropriate.

The present invention will now be described further with reference to the following examples which are illustrative only and non-limiting.

Brief description of the drawings

Figure 1 is a schematic showing the various aspects of the invention.

Figure 2 shows a glycosylation profile of N-linked oligosaccharides released from a single haptogloblin isomer in plasma separated by 2D gel electrophoresis. The glycosylation profile differs between the normal and diseased states.

Figure 3a shows the separation of high molecular weight glycoproteins from sputum by 1D SDS-AgPAGE and PAS stained for carbohydrate content. I – Cystic Fibrosis subject #1 with acute pulmonary exacerbation; II – Cystic Fibrosis subject #1 recovered after antibiotic/antiinflammatory treatment; III – Normal subject; IV – Cystic Fibrosis subject #2 with acute pulmonary exacerbation. V – Cystic Fibrosis subject #2 non-responsive to antibiotic/antiinflammatory treatment.

Figure 3b shows the glycosylation profile of O-linked oligosaccharides released from mucin, in cystic fibrosis and normal subjects. . I – Cystic Fibrosis subject #1

with acute pulmonary exacerbation; II – Cystic Fibrosis subject #1 recovered after antibiotic/antiinflammatory treatment; III – Normal subject. The mass spectra show differences in the relative amounts of the different oligosaccharide ions present, with some structures completely absent.

Figure 4 shows the results of analysis of single ion chromatographs from reversed phase HPLC separation of the glycans in Figure 3. Differences are observed between the samples. For example, the normal sputum sample shows three distinct isomers of the 1331.5m/z ion, whereas the cystic fibrosis sputum sample shows only two.

Examples

Example 1 - Comparison of N-linked glycosylation profile of nomal and ovarian cancer plasma proteins separated by 2D gel electrophoresis

Methods

Human plasma samples were depleted of some well known abundant proteins, and the proteins were separated by 2D gel electrophoresis, and electro-blotted to PVDF membrane. Identical blots were stained with Direct Blue71 (which stains the majority of proteins) and Periodic Acid/Sciffs (PAS) stain (destructive carbohydrate stain). Glycoproteins were identified from the PAS stained membrane and the corresponding Direct Blue71 protein spots were excised from the blot and treated with PNGaseF *O*-linked oligosaccharides were subsequently released by beta-elimination and analysed by ESI LC/MS.

Sample Preparation and 2D-Electrophoresis

Human plasma samples were prepared for 2D electrophoresis by depletion of fibrinogen, IgG (immuno-affinity using immobilized protein G (Amersham Pharmacia), and albumin.

Depleted plasma, precipitated with ethanol, was solubilised, and reduced and alkylated. Reduction and alkylation gives you gels which contain less horizontal (false) isoforms and less dimers/trimers.

Electroblotting to PVDF membrane

Human plasma proteins separated by 2D electrophoresis are electroblotted from the miniChip™ or GelChip™ gels to Immobilon – P^{SQ} PVDF

membrane (Millipore). Identical blots of the protein were stained with Periodic Acid/Schiffs base for glycoproteins and Direct Blue (DB71), as a general protein stain. Potential glycoproteins were identified by PAS and the corresponding Direct Blue stained spots which were identified used for glycoproteins analysis.

PNGase F enzymatic cleavage and purification of N-linked oligosaccharides

PVDF membrane spots were cut from the membrane and placed in separate wells of a 96 well incubation plate. The spots were then covered with 100 µl of 1% (w/w) PVP (polyvinyl pyrrolidone 40'000) in 50% methanol, and the samples shaken for 20 min before being removed and thoroughly washed with water and placed in a clean, dry well. *N*-linked oligosaccharides were then cleaved from the treated protein by the addition of 5 µl of PNGase F (0.5 Units/µl) and incubated overnight at 37°C. The sample wells were surrounded by wells containing water to prevent evaporation and the plate sealed.

Oligosaccharides were collected after sonicating the samples (in the 96 well plate) for approximately 5 min. The released *N*-linked oligosaccharides were transferred from the wells into carbon micro columns.

The carbon micro columns were made from 15 mg of graphitised carbon from an Alltech Carboograph Extract-Clean Tube (Sydney, Australia) which is in methanol, into a ZipTip (Millipore). The Carbon Micro Columns were activated with 3 x 10µl of 90% (v/v) MeCN/ 0.5% formic acid pre-wash, followed by 0.5% formic acid. The *N*-linked oligosaccharides were trapped on the column by sucking up and down the sample several times. The salts were washed of the column using 3 x 10µl of 0.5% formic acid and the *N*-linked oligosaccharides eluted using 2x 10µl of 25% (v/v) MeCN/ 0.5% formic acid and dried under vacuum. Before analysis with LC-ESI-MS the samples were re-hydrated.

β-elimination, chemical release and purification of O-linked oligosaccharides

Each spot was wetted with approximately 2 µL methanol. A solution of 50 mM KOH and 0.5M $NaBH_4$ (20 µL) was added and the spots incubated for 16 hours at 50°C. The samples were neutralised by adding 1 µL Glacial Acetic Acid and transferred into a AG50WX8- cation column (H^+ - form) (BioRad) packed into a Millipore ZipTip, washed with methanol and eluted with 2 x 60µL of water and dried under vacuum. The remaining borate was removed by addition of 50 µl of 1% acetic acid in methanol, followed by evaporation. This was repeated a total of

five times to ensure all the borate was removed. The samples were then re-suspended in 10 µl of H_2O for LC-MS analysis.

LC-ESI-MS of oligosaccharides

LC-MS was used for the analysis of both the *N* and *O*-linked oligosaccharides. The samples were washed onto the home made column (7 µm Hypercarb particles) or Hypercarb column (5 µm Hypercarb particles), both Thermo Hypersil (Keystone Scientific Operations, Runcorn UK) using a Surveyor auto-sampler. An H_2O - acetonitrile gradient (0-25 % Acetonitrile in 30 min, followed by a 3 min wash with 90% acetonitrile) containing 10 mM NH_4HCO_3, was used to separate the oligosaccharides. MS was performed in negative ion mode with three scan events: Full scan with mass range 320-2000 m/z, dependent zoom scan, and dependent MS/MS scan after collision induced fragmentation. Collision conditions used were a normalised collision energy of 40%, activation Q of 0.250 and an activation time of 30 ms. Dynamic exclusion of ions

Results

Figure 2 shows that the N-linked oligosaccharides released from a single haptoglobin isomer separated by 2D electrophoresis results in a different glycosylation profile by mass spectrometric analysis.

Example 2 - Comparison of O-linked glycosylation profile of normal and cystic fibrosis sputum high molecular weight proteins separated by 1D gel electrophoresis.

Materials and methods

Sputum was obtained from healthy and cystic fibrosis patients

Separation of mucins by SDS-AGPAGE

SDS-AGPAGE gels were made by boiling two solutions with 0.5 % agarose and 0.375 M Tris-HCl pH 8.1, one also containing 6 % T, 2.5 % C (piperazine diacrylamide) and 10 % glycerol. The 0-6% gradient polyacrylamide/0.5% agarose gradient gels were cast in the mini-Protean gel casting apparatus (Bio-Rad, Hercules, CA) at 50°C after adding N,N,N',N'-tetramethylethylenediamine (0.0125%) and ammonium persulphate (0.005%) to each solution. The gels were polymerised for 1 hour at 50°C and the agarose was then allowed to set at room temperature over-night in a humidified

environment. The anode and cathode buffer was 192 mM tris-borate pH 7.6 with 1 mM EDTA and 0.1 % SDS.

The sputum was reduced and alkylated in sample loading buffer (Tris-HCl pH 8.1) as described above and sample equivalent to 100 μg Muc2 and 20 μl saliva were loaded onto SDS-AGPAGE gels, and electrophoresed at 100 V for 2-3 hours, until the dye front migrated out of the gel. Proteins were then electroblotted as above, with methanol excluded from the anode buffer. Gels were stained using PAS or Alcian Blue (0.125 % Alcian Blue in 25% ethanol and 10 % acetic acid for 10 min and destained in 100 % methanol for 20 min.

Reductive Alkaline β-Elimination of Oligosaccharides

Oligosaccharides attached to glycoproteins separated by SDS-PAGE or SDS-AGPAGE and blotted to membrane were released by reductive β-elimination. Direct blue or Alcian Blue stained bands were excised from the membrane, wetted with methanol, and incubated at 50°C for 16 hours in 20 □L 50 mM NaOH and 0.5 M $NaBH_4$. The resultant solutions were neutralised by the addition of 1 μL glacial acetic acid, before being desalted with 25 μL AG50WX8 cation exchange resin (Bio-Rad) in a zip-tip (Millipore), and dried in a Savant SpeedVac. Borate was removed as its methyl-ester by repeated (5 times) addition and evaporation of 50 μL 1 % acetic acid in methanol to each sample. Finally the samples were resuspended in 10 μL MilliQ water for LC-MS analysis.

Mass spectrometric identification of released oligosaccharides

Desalted oligosaccharides were analysed by LC-MS/MS on a home-made graphitised carbon column 7 μm Hypercarb particles (Thermo-Hypersil, Runcorn UK) in a 250 μm ID column, after introduction using a Surveyor autosampler. A solvent rate through the column of 5 μL/min was provided by a Surveyor LC pump (ThermoFinnigan, San Jose, CA) with flow splitting from 100 μL/min. Oligosaccharides were eluted with a H_2O-acetonitrile gradient (0-40 % acetonitrile in 30 min, followed by a 3 min wash with 90 % acetonitrile) with constant 10 mM NH_4HCO_3. Mass spectrometry was performed on an LCQ Deca (ThermoFinnigan) in negative ion mode, with three scan events: Full scan with mass range 320-2000 m/z, dependent zoom scan of the most intense ions in each scan, and dependent MS/MS scan after collision induced fragmentation. The capillary temperature was 180°C, the capillary voltage was 32.0 V and the electrospray voltage was 2.5 kV. Collision conditions used were a normalised collision energy of 40%, and an activation time of 30 ms. Dynamic exclusion of

ions for zoom scan for 30 s was introduced after 3 selections within 30 s. For MS/MS the normalised collision energy was 35 % with an activation time of 30 ms.

Results and Discussion

Analysis of Mucin Oligosaccharides.

As compared to smaller glycoproteins, mucins (> 200 kDa) are predominantly glycosylated with O-linked oligosaccharides, with up to 80 % of the weight. Mucins found on mucosal surfaces are supposed to be important interaction molecules due to their glycosylation. Traditionally characterisation of oligosaccharides from mucin is carried out after isolation of mucin fractions with isopycnic centrifugation, followed by gel- and anion exchange chromatography (ref). As a final step the oligosaccharides are released and characterised using mass spectrometry, monosaccharide composition analysis, and ^{1}H-NMR. The approach taken here is using a highly resolving agarose-polyacrylamide composite gel for isolation of mucin fraction, since high molecular weight of most mucins make them unsuitable for traditional SDS-PAGE. Limited characterisation of released oligosaccharides using high resolving LC-MS, provides sufficient information for identifying glycosylation from well defined mucin subpopulations. Another advantage of the methodology is that a lower amount of mucin will be consumed for the analysis i.e. less than 100 μg of a crude mucin fraction, compared to several milligram of purified mucin for the traditional analysis. The utility of the method described here for glycosylation analysis of gel separated mucins has been validated by comparison with previously reported glycosylation profiles of rat mucins.

The method described here profiles the oligosaccharide structures found on a mucin glycoprotein species from healthy and cystic fibrosis patient sputum.

Results:

O-linked glycosylation profiling of oligosaccharides released from mucin separated by 1D AGPAGE gel electrophoresis shows marked differences between CF and normal sputum in both the gel pattern and the glycosylation of the high molecular weight glycoproteins. Figure 3a shows that acute pulmonary exacerbation of two cystic fibrosis patients (I and IV) results in the high molecular weight glycoprotein bands of sputum separating at a apparent lower molecular mass on AgPAGE gels than that of non-CF sputum (III). Interestingly, the

successful treatment of the pulmonary infection with antibiotics and anti-inflammatories results in the glycoprotein bands appearing in the CF patient sputum sample at the same high molecular weight as those of non-CF sputum (II). In addition, the sputum of a CF patient who did not respond to treatment still maintains the appearance of lower molecular mass glycoproteins.

Correspondingly, the total ion mass spectra of the O-linked oligosaccharides released from the high molecular weight glycoproteins (Figure 3b) show differences in the relative amounts of the different oligosaccharide ions present in non-CF compared with CF patients with acute pulmonary infection, with some structures completely absent in the normal sputum (I & III) (e.g m/z 1477.2). The oligosaccharide profile upon treatment of the infection with antibiotics and anti-inflammatories is seen to alter with the treatment (II).

Analysis of single ion chromatographs from the reversed phase HPLC separation of the glycans also shows differences between samples. For example, the 1331.5 m/z ion the normal sputum sample shows three distinct isomers, while only two are detected from the CF sputum sample (Figure 4). These representations of the glycosylation profiles of specific separated proteins, or protein mixtures, can be used separately or in combination to compare different sample types or disease states.

Example 3 - Identification of O-linked oligosaccharide marker(s) of ovarian cancer

Sample preparation

Human ascites (500 µL) from a patient with ovarian cancer was reduced (40 mM DTT, 100°C, 30 min) and alkylated (100 mM IAA, RT, 12 h) in 1 mL of sample loading buffer for high molecular weight gels.

The samples (60 µL) were loaded onto 0-6% polyacrylamide, 0.5 % agarose composite gels, and run at 100 V for 3.5 h, and blotted onto PVDF membranes. These samples have previously been analysed with western blotting and lectin blotting showing the presence of high molecular weight components (> 1 MDa) staining with CA125 antibodies, CA19,9 antibodies and the lectin WGA (sialic acid and GlcNAc). High molecular weight components were also found by staining with PAS and Alcian blue. The Alcian blue stained blot was cut into five bands. The area cut was estimated to include all the components detected by the antibodies and the PAS and Alcian blue stained components from previous experiments. The samples was treated with 0.05 mM KOH/0.5 M $NaBH_4$ to

release attached oligosaccharides, and after desalting and removal of borate the samples were analysed with LC-ESI-MS and LC-ESI-MS/MS.

Results

In Table 1 is a summary of the detected structures. The range is from 3 to 7 oligosaccharide units and the majority of the structures are either sulphated or sialylated, with 1 to 3 of these charge groups attached. Among the smaller ones are the two isomers NeuAcα2-6(Galβ-3)GalNAcol (galactosylated Sialyl-Tn antigen)(structure 1) and NeuAcα2-3Galβ1-3GalNAcol (Sialyl T-antigen) (structure 2) found together with a disialylated structure NeuAcα2-6(NeuAcα2-3)Galβ-3)GalNAcol (Disialyl T-antigen)(structure 6).

In general the glycosylation is similar between the different samples, with a slight tendency towards smaller and less charged oligosaccharides of the high molecular weight components. MS/MS of the oligosaccharides identified some structural features, but was not so successful in assigning sulphated structures. One reason for that is the heterogeneity of these oligosaccharides were for example structures with the composition HSO_3-$Hex_2HexNAc_2$ were found with up to 10 isomers. Many of the structures have never been described before, especially among the sulphated ones, but also among the other ones where the number of isomers was higher then the number of human structures described in GlycosuiteDB.

The presence of highly charged mucin type molecules could potentially prevent the defence mechanism from coping with cancer, since they could bind to E-selectin and inhibit the selectin-dependent recruitment of leucocytes to the tumor site.

A particularly interesting trisialylated oligosaccharide structure was found. It was detected with an $[M-H]^-$-ion at *m/z* 1257.2 ($Hex_1HexNAc_1NeuAc_3$) (structure 14) at a level of 1 % in all the samples analysed. The structure together with the other data in the table implies that this is a novel oligosaccharide, possibly a Trisialylated T-antigen, and the data from MS/MS indicates that at least two sialic acids are linked together. This kind of linkage has so far only been described present in brain tissue on N-linked oligosaccharides from the NCAM glycoprotein.

Potentially all the structures found could be used as a marker for ovarian cancer. The galactosylated Sialyl-Tn antigen, the Sialyl T-antigen, and the Disialyl T-antigen are widespread oligosaccharide structures, while many of the others are more rare and could increase the specificity of a diagnostic tool.

Table 1 Oligosaccharides detected on human ascites from a patient with ovarian cancer

Composition					Structure	Relative Abundance (%)				
DeHex	Hex	HexNAc	NeuAc	Sulph		1	2	3	4	5
	1	1	1		NeuAc-(Hex-)HexNAcol	0.7	1.4	1.2	5.0	1.8
	1	1	1		NeuAc-Hex-HexNAcol	12.4	7.5	6.9	24.7	17.2
	2	2			Hex-(Hex-HexNAc)HexNAcol	1.0	0.9	0.7	2.6	1.9
	2	2			no structure	0.3	0.6	0.2	0.2	0.3
	2	2		1	no structure	4.9	5.4	6.2	3.3	3.1
	1	1	2		NeuAc-Hex-(NeuAc-)HexNAcol	30.4	19.1	25.0	27.8	51.9
	2	2	1		Hex-(Hex-HexNAc)HexNAcol + NeuAc	1.0	0.9	0.5	0.9	0.7
	2	2	1		Hex-(Hex-HexNAc)HexNAcol + NeuAc	4.2	5.5	5.2	5.4	3.8
	2	2	1		no structure	0.6	0.9	1.0	0.8	0.6
	2	2	1		no structure	1.3	1.1	1.0	0.0	ND
	2	2	1	1	no stucture	4.4	4.2	5.3	5.3	2.1
	2	2	1	1	no structure	5.9	5.4	6.5	5.8	3.4
	2	2	1	1	no structure	16.2	15.0	22.1	5.6	5.5
	1	1	3		Hex-HexNAcol+ NeuAc+ $NeuAc_2$	1.2	1.2	1.1	1.4	1.1
1	2	2	1	1	no structure	0.9	0.6	0.8	0.6	0.5
1	2	2	1	1	no structure	ND	0.6	0.4	0.3	ND
1	2	2	1	1	no structure	2.3	2.5	2.5	2.5	1.3
	2	2	2		no structure	1.4	0.5	0.5	0.9	0.7
	2	2	2		Hex-(Hex-HexNAc)HexNAcol + 2NeuAc	2.3	1.3	1.6	1.6	1.7
	2	2	2		Hex-(Hex-HexNAc)HexNAcol + 2NeuAc	8.6	22.6	5.7	4.9	2.2
1	2	2	1		no structure	ND	ND	0.3	ND	ND

1	2	2	1		no structure	ND	1.1	2.4	0.4	0.2
	2	2	2	1	no structure	ND	0.5	1.3	ND	ND
	2	2	2	1	no structure	ND	1.2	1.6	ND	ND
					Sum	100.0	100.0	100.0	100.0	100.0
					Average length (monosaccharide units)	4.9	5.2	5.2	4.4	4.3
					Average charge	1.7	1.8	1.8	1.6	1.7

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are readily apparent to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

DATED this twentieth day of August 2002

Patent Attorneys for the Applicant:

F.B. RICE & CO.

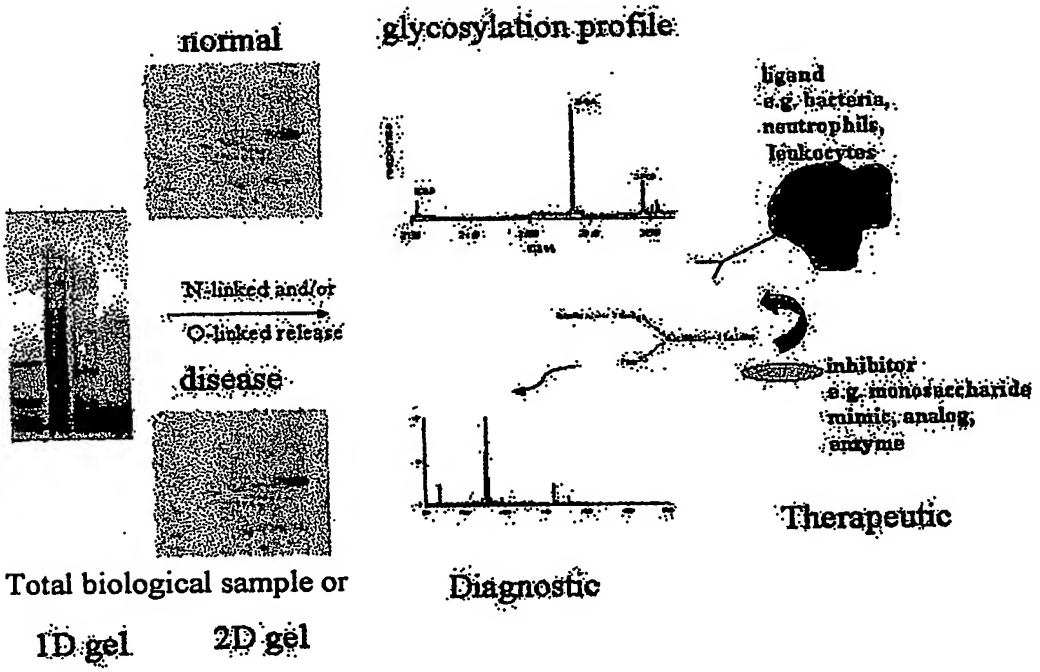
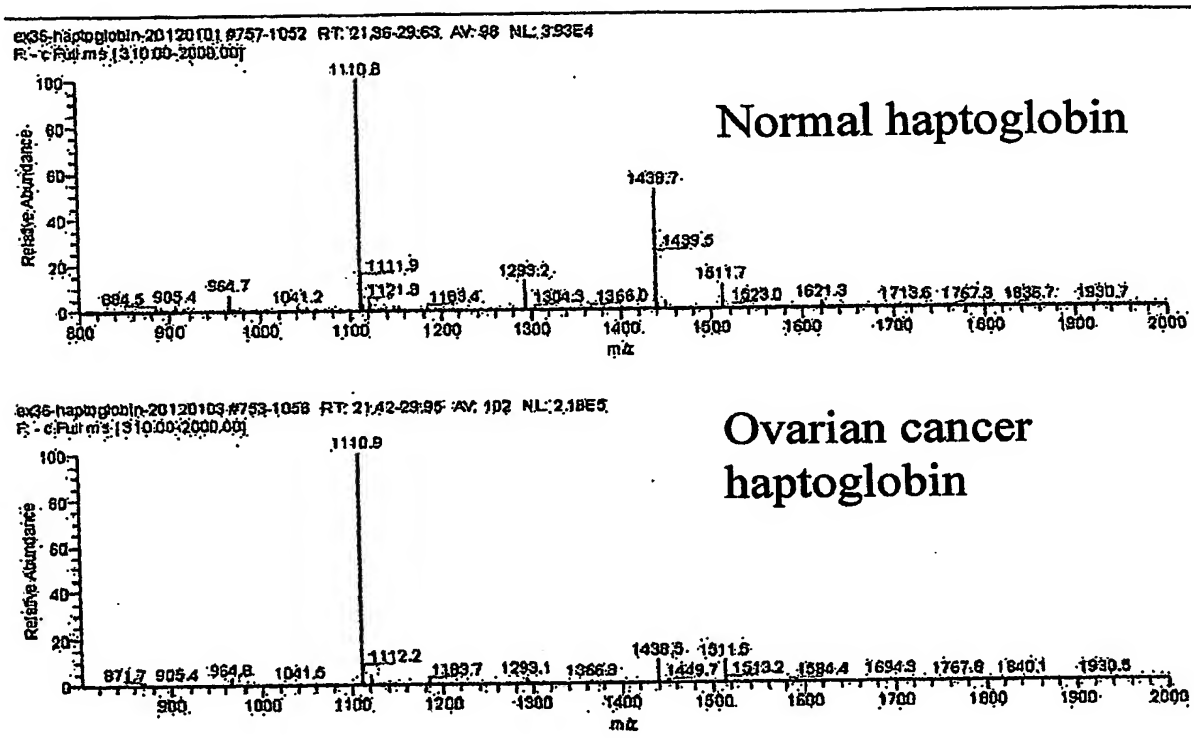

normal
glycosylation profile
ligand
e.g. bacteria,
neutrophils,
leukocytes
N-linked and/or
O-linked release
disease
inhibitor
e.g. monosaccharide
mimic, analog,
enzyme
Therapeutic
Total biological sample or
1D gel
2D gel
Diagnostic

Figure 1

Figure 2


Normal haptoglobin
Relative Abundance
m/z
1110.8
1439.7
1439.5
1111.9
1293.2
1811.7
1121.8
1183.4
1304.3
1366.0
1623.0
1621.3
1713.6
1767.3
1836.7
1930.7
964.7
904.5
905.4
1041.2
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000

Ovarian cancer haptoglobin
Relative Abundance
m/z
1110.9
1112.2
1183.7
1293.1
1366.9
1438.5
1311.6
1449.7
1513.2
1584.4
1694.3
1767.8
1840.1
1930.5
871.7
905.4
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900
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1400
1500
1600
1700
1800
1900
2000

Figure 3A

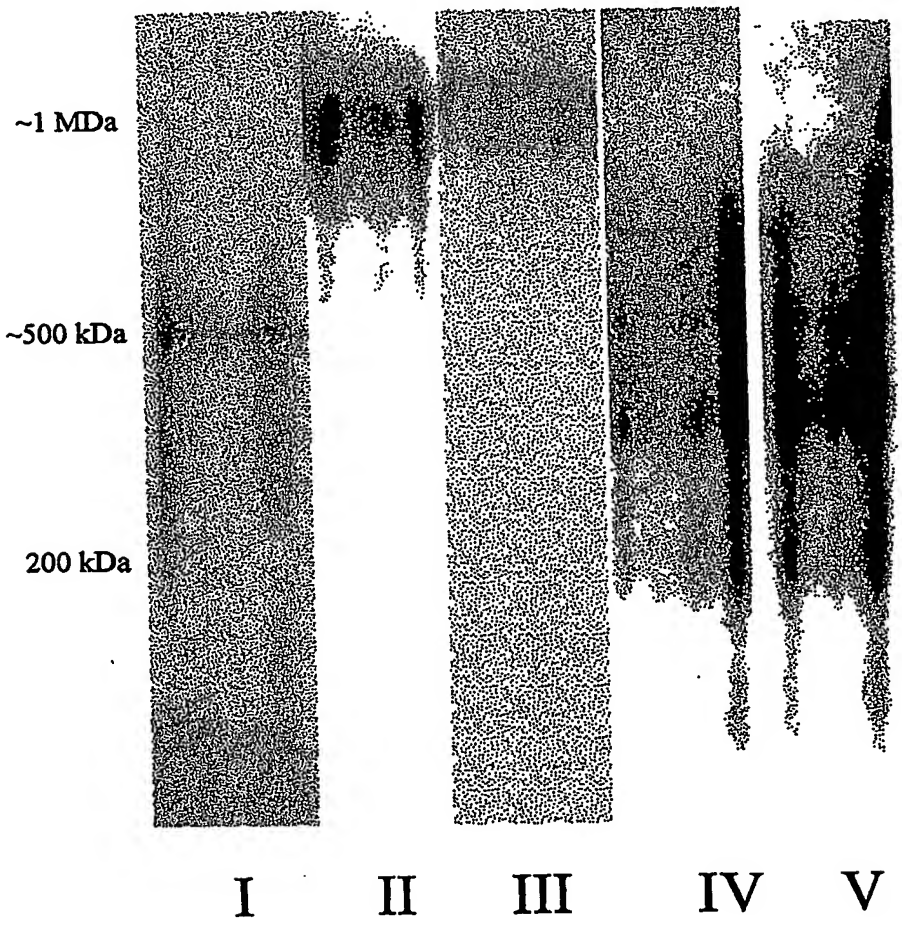


Figure 3B

I: Exacerbated CF patient

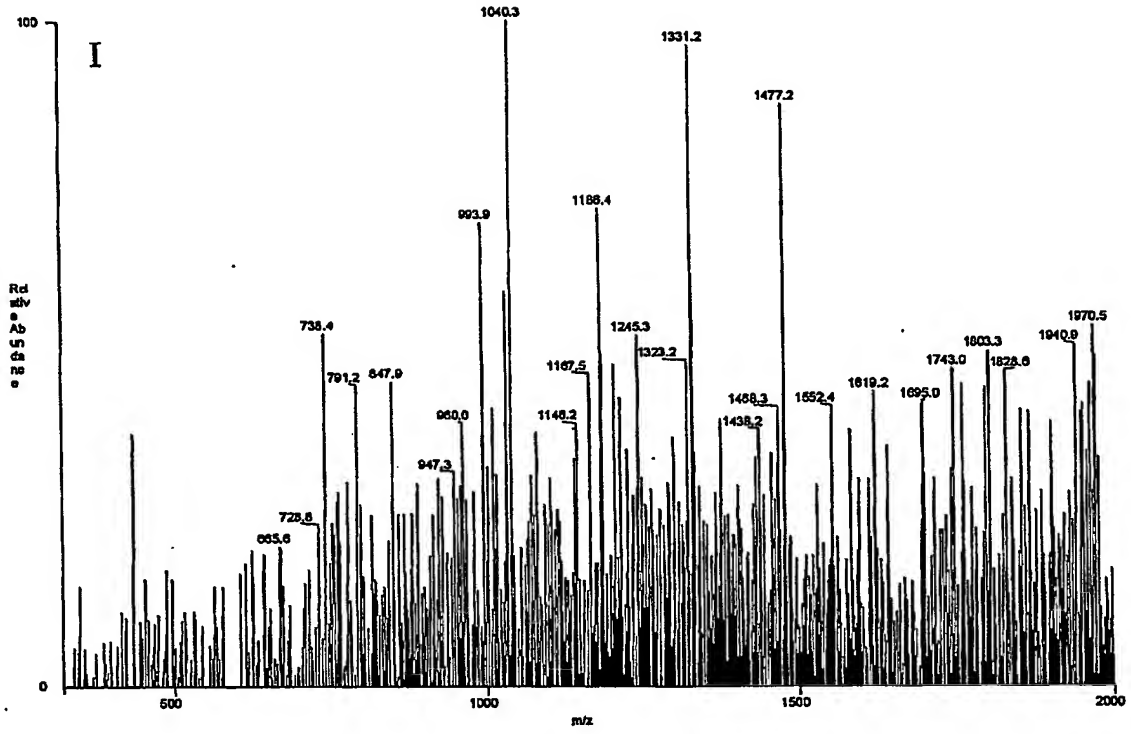


Figure 3B

II: CF patient after treatment

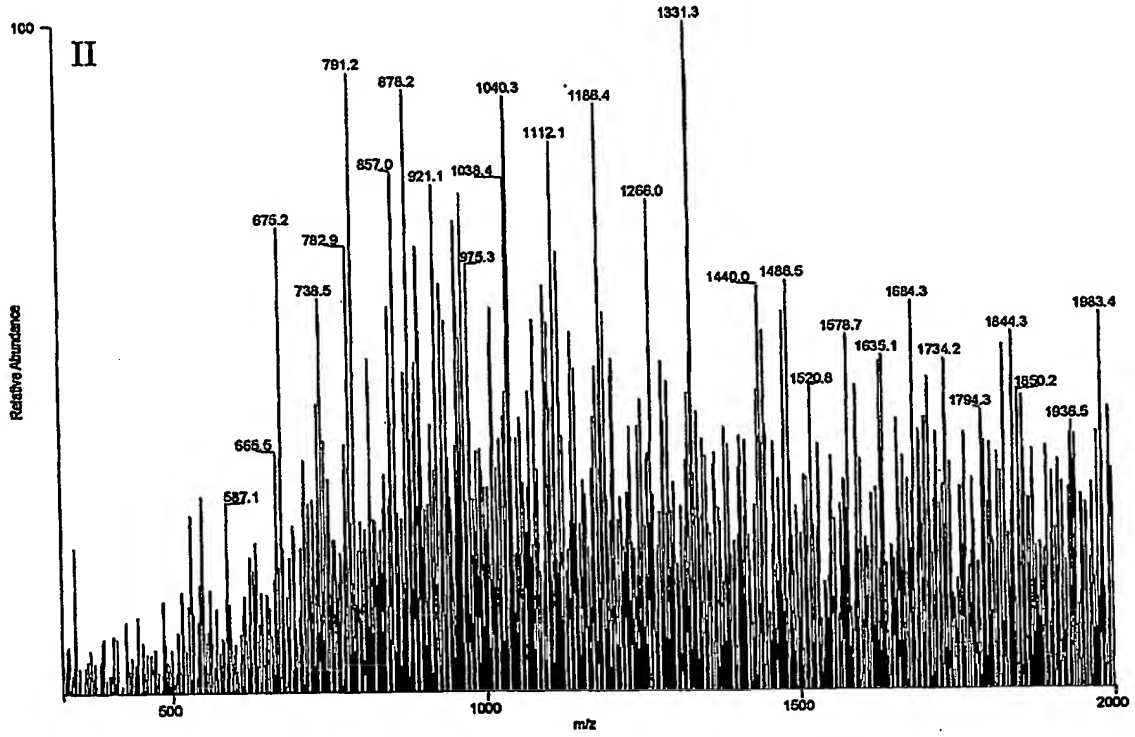


Figure 3B

III: Non-CF sputum

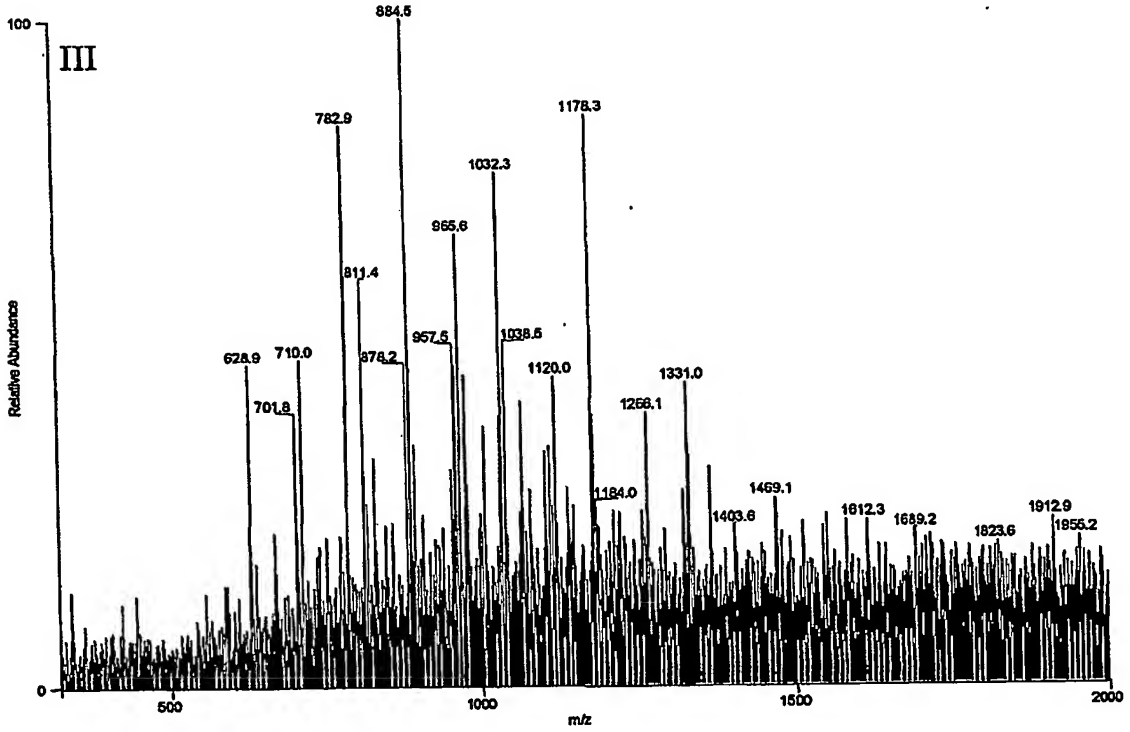
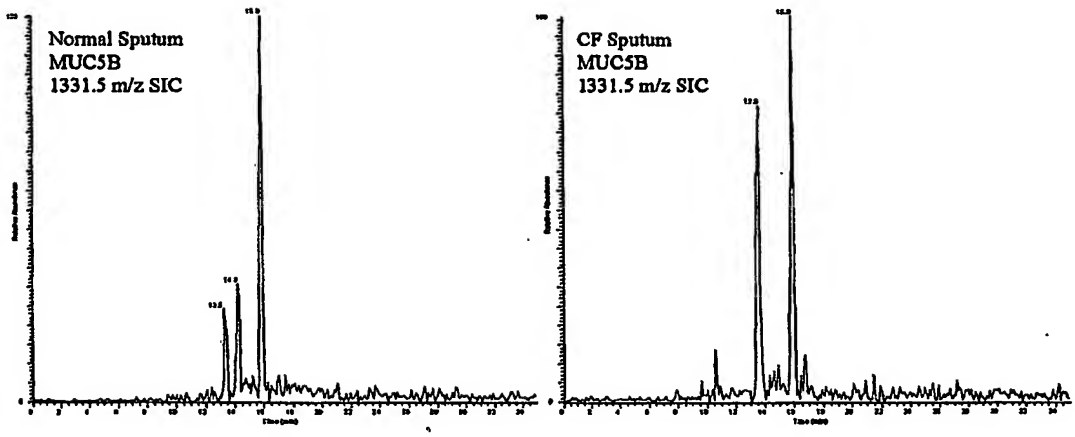


Figure 4


Normal Sputum
MUC5B
1331.5 m/z SIC
CF Sputum
MUC5B
1331.5 m/z SIC

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